

From the Institute of Hygiene, University of Zurich
[Director: Prof. Dr. H. Mooser].

Observations on the L Forms of Proteus and Salmonella

Inaugural-Dissertation

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presented by

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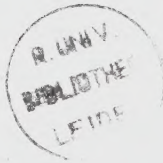
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To my Wife



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Introduction.

In 1935, *Klieneberger* (1) found in cultures of *Streptobacillus moniliformis* that the bacillary chains were interspersed with soft elements resembling a pleuropneumonia-like growth. She designated this component (2) which grew on artificial media and did not revert to the streptobacillary form as L1. Subsequently the work of *Dienes* (3) and others (4, 5, 6, 7 and 8) demonstrated that the L1 was a growth form of this pleomorphic bacillus. Tiny

colonies similar to the L1 were reported from other species of bacteria (9 and 10). *Pierce* (11) in 1942 noted that the pleuropneumonia-like L1 forms were resistant to penicillin. Since then further studies have shown that many gram-negative and gram-positive bacteria developed into similar L1 elements in the presence of this antibiotic (12). Other environmental changes also greatly increased the transformation of bacteria to the L1 growth such as cold, bacteriophage, antibodies (13) and various chemical compounds. In a recent review of the literature by *Dienes* and *Weinberger* (12) the term "L form" replaced L1 and the colonies and cultures are called "L type". This nomenclature has been employed in this publication. It was demonstrated that a pleuropneumonia-like growth resulted when the bacilli of *Proteus* (14 to 19) and *Salmonella* (13, 18 and 20) were in contact with penicillin.

There have been few observations of the pathogenicity of L type growth obtained from *Str. moniliformis* (5, 21), *Flavobacterium* (21), and *Vibrio comma* (22) in mice. The nonpathogenicity of *Salmonella* L type growth for rabbits was shown by *Weinberger*, *Madoff* and *Dienes* (20). In the present paper pathogenic bacillary forms of *Proteus vulgaris* and *Salmonella typhimurium* yielded L type growths after contact with penicillin. When non-stabilized L forms were injected intraperitoneally, the mice died from an infection of the bacillary form. However, a stabilized L form was obtained from *S. typhimurium* which proved non-pathogenic for mice.

MATERIALS AND METHODS.

I. Experiments with Proteus vulgaris.

Initially stock cultures of *Proteus* OX 19 and *Proteus vulgaris* were used. Later more satisfactory results were obtained from freshly isolated *Proteus vulgaris* strains. These were from routine specimens submitted to this laboratory such as urine, feces, pus from an otitis and a lung fistula. All of the 23 strains exposed to penicillin produced L type growth.

The media used to study the transformation of *Proteus* into its L forms consisted of soft ox heart infusion-peptone agar (1.5%) plus 5% beef blood boiled and sterily filtered (23). This was enriched with 30% horse serum. Various concentrations of penicil-

lin (10 to 1000 u./ml.) were added to this transparent agar base when the medium was poured into Petri dishes. The agar squares cut from the plate to be examined were placed between a slide and a cover slip and ringed with petrolatum. These thinly cut agar slices could conveniently be examined under oil immersion. Wet preparations were stained with 1-2 drops of alkaline methylene blue for rapid identification. However, for more detailed cytological study the bacterial surfaces of the agar squares were pressed on cover-slips or slides and lifted off. The resulting stamp was then specially fixed and stained. The following fixatives were used: Schaudinn's fluid, methyl alcohol, Bouin's fluid, Carnoy and Lebrun's fluid, and osmic acid vapor. The agar fixation method of *Klieneberger* and *Smiles* (24) proved satisfactory for colony studies. The stains employed included Giemsa, May-Grünwald, gentian violet, methylene blue, and safranin. The osmic acid-Giemsa and Bouin-Giemsa method as described by *Klieneberger-Nobel* (25) gave excellent results for chromatin and cytoplasmic details. When the osmic acid vapor fixation was followed by Bouin-Giemsa, the preparations obtained were also very satisfactory.

Development of L type Cultures.

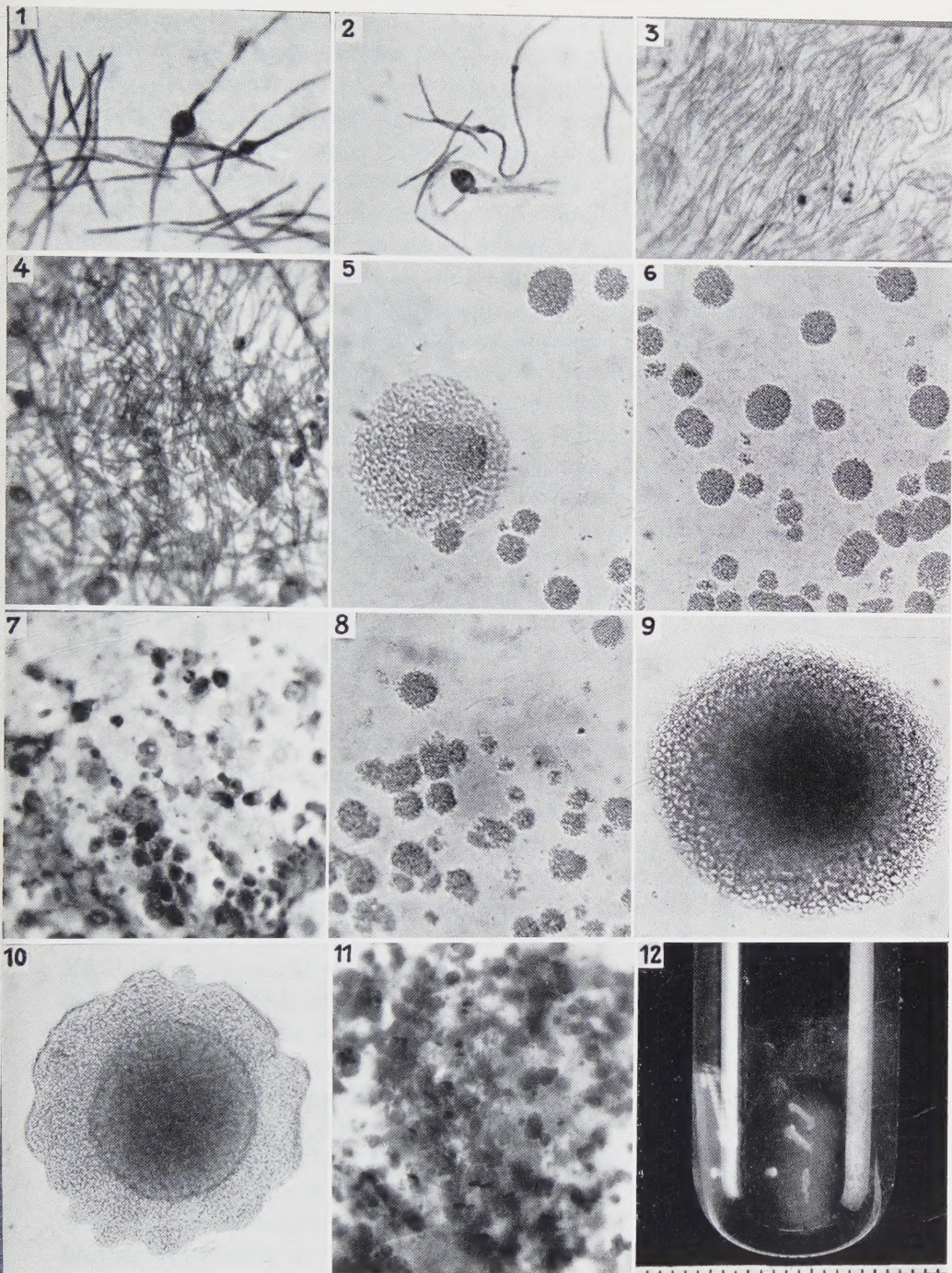
A few drops of a 6 to 8 hours nutrient broth culture of *Proteus* were spread on soft horse serum agar containing 100 to 1000 u./ml. penicillin and incubated at 36° C. aerobically. After 5 to 6 hours incubation, a moist film had formed. Direct microscopic examination revealed that long filaments had replaced the small bacilli. The filaments were most active on the periphery of the growth, where they were arranged as hair-like strands in constant waving motion or at times moving in concentric circles. At this time, several filaments had slight spindle-shaped swellings. Later they contained large axially placed highly refractile spheroids about the size of yeast cells. Less frequently they were present as lateral protuberances. Some of these so-called "large bodies" were in rapid spinning motion, although most were stationary. Occasionally bizarre protozoan-like forms were found. In stained preparations a beaded arrangement of the chromatin material in these filaments could be seen (Plate I, Photographs 2 and 3). The fusiform swellings had the chromatin at one end at first, but later stained more evenly (Photographs 1 and 2). As the bodies grew in size, the con-

taining filaments stained less uniformly. When fully developed, the chromatin reaction was very strongly positive in these bodies and the filaments broke into fragments (Photograph 2). These disjointed sections of the filaments appeared as empty shells. At the end of 24 hours the number of large bodies lying freely on the surface of the media had increased greatly and a mass of long filaments was seen at the periphery of the inoculum. Toward the center of the growth the large bodies appeared to increase in number and a meshwork of fewer filaments was present (Photographs 3 and 4). A moderate number of very small transparent

Explanation of Plate I.

Photomicrographs. Numbers 1 to 7 illustrate the development of *Proteus* into filaments containing large bodies and their transformation into L type colonies after contact with 1000 u./ml. of penicillin. Numbers 8 to 12 are of *Salmonella typhimurium* L type colonies grown anaerobically without penicillin, except for no. 9 which contained 100 u./ml. Numbers 5, 6, 8, 9, 10 and 12 were made from unstained preparations.

- 1) *Proteus* after 6 hours incubation on agar containing 1000 u./ml. of penicillin showing swollen filaments in various stages of large body formation. Osmic acid, methylene blue, $\times 1200$.
- 2) Same preparation as no. 1 demonstrating completely formed intensely staining large body in a fragmented achromatic filament. $\times 1200$.
- 3) Waves of beaded long filaments after 24 hours incubation on the periphery of the inoculum. Osmic acid, May-Grünwald, $\times 500$.
- 4) A network of filaments and large bodies occurring freely toward the center of the inoculum after 24 hours incubation, Carnoy-Lebrun, Giemsa, $\times 1200$.
- 5) Primary isolation showing a large L type colony with dark center and several small transparent L type colonies after 2 days incubation. Unstained, $\times 100$.
- 6) Well adapted small uniform L type colonies with some showing fusion. Note similarity to *Salmonella* colonies in no. 8. Unstained, $\times 100$.
- 7) Markedly pleomorphic organisms constituting a small L type colony after 2 days incubation. Methylene blue, $\times 1200$.
- 8) Tiny uniform L type colonies of *S. typhimurium* grown anaerobically after 3 days without penicillin. $\times 100$.
- 9) Well developed L type colony with dark center after 1 week incubation on agar containing 100 u./ml. of penicillin. Note also highly refractile spherical L forms on colony's edge. Unstained, $\times 500$.
- 10) Typical 2 week-old L type colony demonstrating dark center with halo and scalloped border. $\times 500$.
- 11) Spheroid organisms and granules constituting a small *Salmonella* L type colony after 3 days incubation. Note similarity to no. 7 of *Proteus*. Carnoy-Lebrun, May-Grünwald, $\times 1200$.
- 12) Agar transplants of L type colonies in horse serum broth showing warty growth jutting into fluid and penetration into agar after 2 weeks incubation anaerobically. The lower layer of agar on the side of the tube shows the depth of growth before separation of the 2 pieces. Note wad of cotton fibers used to support growth. $\times 2$.



colonies appeared after 1 to 2 days. These were slightly embedded in the media and approximately 0.5 mm. in size. These adherent colonies grew densely and later became opaque white with a brownish center. Their diameters increased slowly, as they continued to grow into the depths of the agar. After 2 weeks they measured about 2 mm. Fusion occurred in these uniform well adapted pleuropneumonia-like colonies in contact with penicillin (Photograph 6). A few much larger colonies with definite halo appearance and denser centers were present after 24 hours incubation following primary isolation (Photograph 5). These colonies usually became granular and in 1 to 2 days produced swarming filaments and bacilli. The microscopic examination of cut out agar squares showed that the filaments were no longer present in the well adapted colonies. These colonies consisted of pleomorphic spheres with the general morphology of large bodies (L forms). The forms included large bodies with deeply staining masses of chromatin in the center, some with granules $2-3\ \mu$ in size, a moderate number of very small coccoid forms less than $1\ \mu$ and several poorly staining autolysed spheres. Some of the organisms stained faintly with methylene blue and pink with Giemsa and May-Grünwald, whereas other cells stained purple-blue (Photograph 7).

L type Colony Growth and Subcultures of Proteus.

Since these L type colonies grew into the media, transplants were made by cut-outs from the agar which were pressed growth-side down, smeared on the new surface and left there. The new growth then appeared on the surface of the agar and under the cut-out squares. Such subcultures were made at intervals of 2 to 5 days. The larger the concentration of penicillin, the longer it took for the L type growth to revert to bacilli when not subcultured. Thus, for example, L type colonies on agar containing 1000 u./ml. of penicillin could be safely transferred at weekly intervals while on media with 10 u./ml. of this antibiotic swarming occurred in 2 to 3 days, necessitating daily transfers. Therefore, it was possible to maintain L type growth in the presence of penicillin for 8 months provided transfers were made at appropriate intervals. The Petri dishes were arbitrarily divided into 3 sections so as to grow 3 different strains on 1000 u./ml. of penicillin with weekly transfers. This procedure proved to be a saving of materials and

time. L type colonies, when subcultured to higher or lower concentrations of penicillin than that used for the initial isolation, appeared unchanged in characteristics. In an attempt to stabilize the L type colonies without penicillin, single colonies were isolated under the binocular dissecting microscope. Subcultures were made in serially descending concentrations of penicillin from 1000 u./ml. to none or abruptly dropped from 1000 u./ml. to none. These L type growths reverted to bacilli when transferred to plates without penicillin in 12 to 18 hours or after several daily transfers which rarely exceeded 1 week. Without penicillin under anaerobic conditions the L type growth likewise returned to bacilli in 1 to 2 days. Transplants of cut-outs from plates containing various concentrations of penicillin to nutrient broth resulted in a slight cloudiness and small motile bacilli appeared in 3 to 5 days.

L type cultures containing 1000 u./ml. of penicillin were incubated for 2 days and then placed in the refrigerator at 5° C. for 5 weeks. Upon subculture to enriched agar containing penicillin, followed by incubation (36° C.) the L type colonies grew. When the refrigerated growth was transferred to plates with no penicillin, swarming occurred after 1 to 2 days. These resultant bacilli were as sensitive to penicillin as their original parent *Proteus* strains and could be transformed to L type colonies when in contact with this antibiotic.

Filtration of the L type Growth of Proteus.

Several series of plates containing 100 and 1000 u./ml. of penicillin were inoculated from broth cultures. The surface growth was scraped with a cotton swab while the L type colonies were obtained from the minced agar or mortar and pestle maceration with 3 to 4 ml. of broth. This emulsion was placed in a small unglazed porcelain filter and allowed to filter spontaneously or hastened by gentle negative pressure. The clear filtrate was pipetted onto plates with and without penicillin. The emulsions were prepared after different periods of incubation, namely 7 hours, 1, 2 and 5 days. These produced consistently negative results.

In 3 instances the filtrates prepared from a 7-day growth on agar with 100 and 1000 u./ml. of penicillin yielded L type growth on media containing penicillin and *Proteus* bacilli on media without this antibiotic. It is to be noted that some of these one-week-old cultures had shown signs of swarming when filtered and that

one was from a mixed growth of 2 different strains. Nevertheless the filtrates from the resultant bacillary growths proved sterile.

Since the L type colonies appeared to grow into the media, it was thought that this mechanism could be used to test their filtrability. Small square cut-outs from 2 to 3 days growth on enriched agar containing 1000 u./ml. of penicillin were placed growth downward on a membrane filter disk 2 cm. in diameter (Membranfiltergesellschaft, Sartorius-Werke AG., Göttingen). These thin disks prepared from cellulose esters were then placed on serum agar also containing 1000 u./ml. of penicillin. After 1 week, the disks were removed and the agar under them examined for growth. Membrane filters of graduated pore size, i.e., "coarse" 3-0.75 μ , "medium" 0.75-0.50 μ , "fine" 0.50-0.20 μ and "very fine" less than 0.2 μ were used. A complete duplicate of the L type growth was seen under the coarse filter and a few colonies were present under the medium filter. No growth was seen under the fine and very fine membrane filters. The newly formed L type colonies from organisms which had passed through the filters produced *Proteus* bacilli when transferred to agar without penicillin.

Animal Inoculations with Proteus L Type Growth.

Saline emulsions of L type growth were prepared from 2- to 5-day-old heavily seeded agar cultures containing none, 50 and 100 u./ml. penicillin. The granular milky product was run through gauze to remove the agar pieces and used immediately. It was injected intraperitoneally into white mice in 0.2 ml. amounts. These animals died within 4 to 7 days after inoculation with the L type growth. Smears and cultures of the peritoneal exudate yielded *Proteus* bacilli.

In another experiment, the mice inoculated with a suspension of the L growth of *Proteus* remained well during the 5 days of observation. After this period, a liver emulsion from one of these mice was injected intraperitoneally into new mice; the latter succumbed from a *Proteus* bacillary infection.

As a comparison, mice were injected in a similar manner with *Proteus* bacilli. These animals died in less than 24 hours.

II. Experiments with Salmonella typhimurium.

The strain of *S. typhimurium* used in these experiments was isolated recently in this laboratory from a patient's stool. The

methods were similar to those employed in the experiments with *Proteus*. A few drops of a 6 hours nutrient broth culture were spread on soft horse serum agar plates containing penicillin and incubated at 36° C. anaerobically. Older broth cultures produced too heavy a growth with resultant crowding. Concentrations of penicillin from 10 to 10,000 u./ml. were employed. The alkali-pyrogallic acid method was used to obtain anaerobiosis.

During the first day of incubation, occasional slightly swollen filaments were seen. This stage of large body formation was not as clear as that noted in *Proteus*. In addition there were seen some highly refractile spheroid L forms 10 to 20 μ , some amoeboid-shaped and others which were larger containing granules. After 1 to 2 days, many pin point transparent L type colonies were present, especially on the periphery of the inoculum. Toward the center of the inoculum, a few 3 mm. large opaque white colonies appeared after 24 hours. These larger colonies contained some large bodies, filaments and bacilli. However, when these anaerobic cultures from the primary isolation were left aerobic for 2 to 3 days, the L type growth also became opaque white and when examined revealed long filaments and motile bacilli. These colonies were similar to those obtained from penicillin containing cultures maintained aerobically from the start. In general there were much fewer colonies on the media with penicillin than without it. The L type colonies were further characterized by their growth into the depths of the media. When subcultures by cut-outs containing the small colonies of this primary isolation were made in less than 1 week to media without penicillin bacilli resulted. Also many colonies autolysed during this adaptive period. Ten, 50 and 100 u./ml. of penicillin were found satisfactory for the isolation and maintenance of the L type growth, while larger amounts resulted in poorer growth. A more stabile growth was obtained after 1 to 2 weeks exposure to the antibiotic.

L type Colony Growth and Subcultures of Salmonella.

Once adapted to the media the L type colonies were then cultured anaerobically with and without penicillin. They did not revert to bacilli. As may be observed in Plate I (Photograph 8), the colonies were about 1 mm. in diameter and uniform in appearance after 3 days incubation. Fusion of these colonies occurred where their borders came in contact. As the 1 week-old growth continued

into the depths of the media, the central portion of the L type colonies had a darker appearance (Photograph 9). After several transfers no appreciable difference was seen between the colonies grown in contact with penicillin and those without it. In general, the colonies grew slowly after the first week and rarely became larger than 2 to 3 mm. The older L type colonies were more opaque, light brown in color and had a scalloped edge. The characteristic halo appearance of these pleuropneumonia-like colonies is clearly (Photograph 10) demonstrated in a photograph of a 2 week-old unstained specimen. This well adapted L type growth, when exposed to the air for several hours, still contained viable organisms, but after an exposure of 1 to 2 days they could not be subcultured. In this manner, L type growth was maintained for 9 months.

The spheroid L forms constituting these colonies were readily seen by microscopic inspection (Photograph 9). Stained preparations showed marked pleomorphism, the typical L forms, and very small elementary body-like structures. No bacillary forms were seen (Photograph 11).

The L type growth of 2 to 3 days on horse serum agar without penicillin was transferred on small cut-outs to 5 ml. of horse serum broth in test tubes and incubated anaerobically. The test tubes were prepared for anaerobiosis by layered alkali-pyrogalllic acid and rubber stoppered. Neither agar nor penicillin were used in this broth. The tiny transparent colonies increased in size rapidly during the first week. They became whitish and projected out into the broth as small warts with flattened tops. After 2 weeks the colonies were 2 to 3 mm. in size including their growth into the agar transplant (Photograph 12). This granular growth was cohesive and firmly adhered to the agar without spreading into the broth. When an occasional colony became detached, it settled to the bottom of the test tube where it remained as a granular sphere without appreciable tendency to grow larger. It was thought the colonies in the broth might grow if supported mechanically. Therefore, a mesh of non-absorbent cotton, solid and liquid portions of agar were added to the broth (Photograph 12). No growth appeared on any one of these supports. However, when fresh pieces of enriched agar came into contact with the colonies of the original agar transplants, growth also appeared on the former. A slight increment in their size continued for 4 to 5 weeks and the

colonies became more intense brown in color. This pleuropneumonia-like growth of these submerged agar cultures remained viable for at least 12 weeks. The subcultures were made by transferring all or part of the agar transplant with a blunt tipped pipette to agar plates without penicillin. With the aim of furthering the growth in these test tubes, the old broth was replaced by new at various intervals. The fresh fluid was pipetted directly against the surface of the growth. The colonies were rarely washed off into the media. No noticeable improvement of growth was observed. These cultures were viable after refrigeration at 5° C. for 3 months. Attempts to reculture lyophilised organisms have been unsatisfactory.

The depth of penetration of the L colonies was observed on plates poured in 2 separate layers and inoculated. At various times pieces were cut out, the two layers separated and placed into serum broth. In this way it was observed that these colonies had entered the agar to a depth of 3 mm. within 1 to 2 weeks (Photograph 12).

Since the addition of sodium thioglycollate favors the growth of anaerobic organisms it was incorporated in small amounts (0.1 g./l.) into nutrient broth. Transplants were made, as discussed previously, to test tubes containing this fluid media and cotton-plugged. However, better growth was obtained when Brewer's thioglycollate medium containing 0.5 g./l. of this reducing agent, as suggested by *Weinberger et al.* (20), was used. During the first week, the colonies grew to about pinhead size on the surface of the agar transplant and showed fusion. During the following weeks the creamy white colonies became brownish, slightly more rough and lifted into the media, but did not protrude as well as those in horse serum broth. Paper strips containing lead acetate inserted into tubes with L type growth were blackened on the tips after 1 to 2 weeks incubation. The parent bacilli tested in the same medium changed the strips entirely black in 1-2 days. Thus, the pleuropneumonia-like cultures of *S. typhimurium* and the bacilli showed a positive reaction for hydrogen sulfide production. Other studies (20) of the carbohydrate metabolism and gas production of these 2 forms also demonstrated their similarity. In this medium, the L type colonies remained in contact with the agar and did not grow out into the broth. Subcultures from these tubes were viable for at least 6 to 8 weeks. They kept for equally long periods when refrigerated at 5° C.

The application of the so-called shake culture agar with antibiotics was employed by *Grumbach* (26) to test the levels at which the various organisms grew. He demonstrated that in the presence of small amounts of streptomycin the growth of *S. typhimurium* occurred in the anaerobic part, while inhibition appeared in the aerobic portion of the agar. In a similar manner, test tubes containing nutrient agar without dextrose, but with various concentrations of penicillin 0.01 to 1000 u./ml. were inoculated while still liquid with a fresh culture of *S. typhimurium* and solidified rapidly in cold water. After 1 to 2 days incubation, bacilli grew on the surface and throughout the media with low concentrations of this antibiotic. However, with 50 and 100 u./ml. of penicillin anaerobic growth occurred at a level 5 mm. below the surface of the agar and lower. Similar small rough deeply growing colonies, but fewer, were in 200 and 300 u./ml. and rarely did any growth appear in higher concentrations. The spherical colonies had a darker center and grew to about 2 mm. after 1 week. The cylinder of agar was forced out into a sterile Petri dish and thinly cut at various levels. The microscopic examination of wet and stained preparations revealed the colonies to be L type and they consisted of L forms. These slices when transplanted to enriched agar plates and cultured anaerobically produced L type colonies under the center of the transplant, but poorly motile bacilli around its border. Shake cultures without penicillin yielded growth throughout the media. When sparsely seeded the isolated colonies appeared as semiopaque white biconvex disks 2 to 3 mm. large consisting of bacilli.

Animal Inoculations with the Stable L Strain of Salmonella (LST).

The well adapted L type strain which had been on enriched medium without penicillin for 3 to 8 weeks was used to inoculate mice. Suspensions were prepared in the manner described for *Proteus* and in addition from horse serum broth cultures. It was not possible to obtain a uniform suspension as it remained coarsely granular and easily settled out. Ten white mice were injected intraperitoneally with 0.2 ml. These mice showed no immediate ill effects. The five autopsied 1 week later had no gross pathological findings and smears of the peritoneal washings were negative. The other mice remained in good health 3 weeks after the inoculation. Then those previously injected and 2 control mice were in-

oculated with 0.2 ml. of a fresh culture of the parent strain of *S. typhimurium* bacilli. All died 18 hours later. *S. typhimurium* was isolated from the peritoneal exudate. The striking difference between the pathogenicity of the L type growth and the bacilli from, which they had originated, was clearly demonstrated.

Mooser showed that the intraperitoneal growth of murine PPLO is enormously enhanced when PPLO is simultaneously inoculated with the virus of ectromelia (27). He obtained similar results with human strains of PPLO in mice (28). Due to the morphological and cultural resemblance between L forms and PPLO, *Mooser's* method was tried with our strain (LST).

A series of 10 white mice were simultaneously inoculated with the virus of ectromelia and LST. All died in 3 to 4 days from ectromelia. Smears from the peritoneal exudates did not show any organisms. Secondary passages of peritoneal washings and liver emulsion from one of the animals caused death after 3 days from ectromelia. There was no evidence of an infection with LST and smears and cultures remained negative.

DISCUSSION.

It has been shown that in certain pleomorphic organisms the L type growth may occur spontaneously (1 and 29). However, marked changes in the form of bacteria were brought about by alterations in the milieu, usually toxic in nature, such as unfavorable pH, glycine, dl-methionine, 1-phenylalanine (12), and carboxylmethoxylamine (13). Since many L forms occurred at the contact zone of 2 different spreading strains of *Proteus*, this suggested a possible sexual phase to *Dienes* (29). However, no union of these bodies was seen. *Klieneberger* (30) maintained that stimuli which induced bacteria to dissociate also set the L cycle in action. In this work, the transformation into L forms of 23 *Proteus* strains and *S. typhimurium* was aided by contact with penicillin in soft serum agar. The changes were followed more readily in *Proteus* where the motile filaments, not the bacilli, swelled in places to form large bodies. The regularly beaded arrangement of chromatin gave the elongated swarming filaments the appearance of streptobacilli.

The various concomitant changes of nuclear and cytoplasmic structure of *Proteus* in the presence of penicillin were described by *Fleming et al.* (19), and others. The bulbs were more densely

staining than the rest of the filament and produced a strong chromatin reaction. This process is not believed to be due to a simple "plasmoptysis". The author is inclined to concur with *Dienes* (29) that the contents of the filament flow out and produce the large body within a few minutes. As may be seen in Photograph 2, the resultant large body formed in a disintegrating, faintly stained and segmented filament. It has been suggested (30) that the L cycle be regarded as a process of regeneration, probably involving gene recombination. These actively motile large bodies were protozoan-like and contained flagella (19).

After 1 to 2 days incubation, there appeared numerous tiny transparent colonies in the agar. Other colonies were observed that became 2 mm. large, granular, creamy white and which later contained a few large bodies, filaments and finally bacilli. The latter colonies which regularly reverted to bacilli were present in the primary isolations of both *Proteus* and *Salmonella* with penicillin. However, when once adapted to the media, a uniform pleuropneumonia-like growth was maintained for many months. It is the belief of most of the workers in this field that the large bodies reproduce small forms and this pleomorphic growth constitutes the L type colonies. The L forms have been studied with dark field, phase microscopy and oblique incident illumination. *Smith, Hillier and Mudd* (31) used electron microscopy to observe their morphology. They noted that the L forms had a delicate cell membrane as compared with the rigid cell wall of the parent bacilli. Although the L type growths are difficult to stain, excellent results were obtained by using the methods employed for the pleuropneumonia group (25 and 32). The author incorporated some of the rapid fixatives which had been used for nuclear studies in amoebae (33) with satisfactory results.

These stable L forms have practically the same metabolism (12, 15 and 20) and serology (20 and 34) as their parent forms. Although the metabolic rate of the former appeared to be much slower it was manifested by the difference in the rates of production of hydrogen sulfide. Also they are more resistant to penicillin than their bacillary components. Therefore, despite the marked resemblance of these elements with the pleuropneumonia-like organisms, they are considered a growth phase and not a symbiont genetically different from the bacilli. The findings of others (12, 15 and 19) as well as those of the author show that *Proteus* L

type growth reverted to bacilli when transferred to media without penicillin. Similar results were obtained when penicillinase was added to the media. This reversion of *Proteus* L type growth to bacilli is further evidence that the *Proteus* bacillus was its parent form. However, when *S. typhimurium* was grown anaerobically in the presence of penicillin, a stable L strain (LST) was obtained which did not revert to the original bacillary form when the antibiotic was withdrawn. The experimental findings that the *Salmonella* L type growth was non-pathogenic is in accordance with the observations of others (20). The pathogenicity also had disappeared in L forms tested from other bacteria (5, 12 and 21). However, *Pleuropneumonia bovis* and other members of this group listed by *Sabin* (35) are highly pathogenic and host specific. Yet *S. typhimurium* is a natural pathogen of mice. The isolation of pleuropneumonia-like (L) organisms from the human genital tract has been noted (36 and 37). It has been shown that the virus of ectromelia greatly enhanced the *in vivo* growth of murine pleuropneumonia-like organisms (27). However, this virus did not produce a more favorable milieu for the L type growth when injected intraperitoneally. In the mice the L forms of *Proteus* reverted to bacilli and the latter were as pathogenic as the parent bacilli.

Proteus bacilli and freshly isolated L forms did not pass through unglazed porcellan filters. However, emulsions of cultures which were on penicillin for a week and had begun to swarm passed through these filters. The filtrate yielded L type growth on media with penicillin and bacilli on agar without the antibiotic. *Klieneberger* (38) found that the L1 organism of *Str. moniliformis* from old established strains passed through gradocol filters with pore size of 175 to 250 millimicrons. This means that the filterable granules are about the same order of magnitude as the large viruses or the size of organisms of the pleuropneumonia group (35).

Summary.

In media containing penicillin L type growth was obtained from 23 strains of *P. vulgaris*. In spite of a long series of subcultures the L colonies invariably reverted to the bacillary form when transplanted to media without penicillin. Reversion also took place in mice intraperitoneally inoculated with an emulsion of L colonies.

The observations of *Dienes* on *S. typhimurium* are confirmed. A stabilised L type growth was obtained. It grew anaerobically and was non-pathogenic for mice.

Zusammenfassung.

23 Stämme von *P. vulgaris* zeigten in penicillinhaltigem Milieu L-Form. Trotz einer langen Serie von Subkulturen entstanden aus den L-Kolonien bei Rückimpfung auf penicillinfreie Nährböden immer wieder bazilläre Formen; dasselbe war nach intra-peritonealer Impfung von Mäusen mit Aufschwemmungen von L-Kolonien der Fall.

Die Beobachtungen von *Dienes* an *S. typhimurium* werden bestätigt. Es konnte eine stabile L-Form erzielt werden, die anaerob wuchs und für Mäuse apathogen war.

Résumé.

Une croissance du type L a été obtenue sur des milieux contenant de la pénicilline à partir de 23 souches de *Proteus* vulg. Malgré une longue série de passages, les colonies L reprennent la forme bacillaire dès qu'elles sont transplantées sur un milieu dépourvu de pénicilline. Cette réversion apparaît également chez la souris inoculée i.p. avec une émulsion de colonies L.

Les observations de *Dienes* sur *S. typhimurium* ont été confirmées. Une forme L stable a été obtenue, qui se développe en anaérobiose et qui n'est pas pathogène pour la souris.

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